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Complement-mediated Damage to the Glycocalyx Plays a Role in Renal Ischemia-reperfusion Injury in Mice

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Background. Complement activation plays an important role in the pathogenesis of renal ischemia-reperfusion (IR) injury (IRI), but whether this involves damage to the vasculoprotective endothelial glycocalyx is not clear. We investigated the impact of complement activation on glycocalyx integrity and renal dysfunction in a mouse model of renal IRI. **Methods.** Right nephrectomized male C57BL/6 mice were subjected to 22 minutes left renal ischemia and sacrificed 24 hours after reperfusion to analyze renal function, complement activation, glycocalyx damage, endothelial cell activation, inflammation, and infiltration of neutrophils and macrophages. **Results.** Ischemia-reperfusion induced severe renal injury, manifested by significantly increased serum creatinine and urea, complement activation and deposition, loss of glycocalyx, endothelial activation, inflammation, and innate cell infiltration. Treatment with the anti-C5 antibody BB5.1 protected against IRI as indicated by significantly lower serum creatinine ($P = 0.04$) and urea ($P = 0.003$), tissue C3b/c and C9 deposition (both $P = 0.004$), plasma C3b ($P = 0.001$) and C5a ($P = 0.006$), endothelial vascular cell adhesion molecule-1 expression ($P = 0.003$), glycocalyx shedding (tissue heparan sulfate [$P = 0.001$], plasma syndecan-1 [$P = 0.007$], and hyaluronan [$P = 0.02$]), inflammation (high mobility group box-1 [$P = 0.0003$]), and tissue neutrophil ($P = 0.0009$) and macrophage ($P = 0.004$) infiltration. **Conclusions.** Together, our data confirm that the terminal pathway of complement activation plays a key role in renal IRI and demonstrate that the mechanism of injury involves shedding of the glycocalyx.

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Ischemia-reperfusion (IR) injury (IRI) is an unavoidable episode in numerous clinical settings, including ischemic acute kidney injury, myocardial infarction, and stroke, and has systemic manifestations that contribute to multiorgan failure.[1] In transplantation, IRI can have profound detrimental effects on short- and long-term graft functions. The mechanisms involved in IRI are complex and incompletely understood. A number of factors contribute to the pathogenesis of IRI, in which local upregulation and activation of the complement cascade, an arm of the innate immune system, play a key role.[2]

The complement cascade has 3 initiating pathways (classical, lectin, and alternative) that converge to activate a common terminal pathway that results in the formation of the C5b-9 membrane attack complex (MAC). The anaphylatoxins C3a and C5a mediate activation of inflammation by inducing cytokine production, immune and endothelial cell activation, adhesion molecule (eg, vascular cell adhesion molecule-1 [VCAM-1]) upregulation, and increased vascular permeability.[3] The MAC mediates innate cell infiltration and proinflammatory cytokine production and directly causes cell activation or lysis.[4,5] Thus, complement-related tissue injury may be induced by the terminal complement complex C5b-9, by cell-bound ligands, including C4b and C3b, and by circulating C3a and C5a.

Endothelial cell activation, also known to be involved in IRI, is characterized by shedding of the glycocalyx

and increased expression of adhesion molecules and release of damage-associated molecular patterns (such as heparan sulfate [HS], heat shock proteins, and high mobility group box-1 [HMGB1]), with consequent activation of the plasma cascade systems.[6] The endothelial glycocalyx is a negatively charged mesh-like hydrated structure covering the luminal surface of endothelial cells. Proteoglycans like syndecans 1 and 4 and glypican 1, with bound glycosaminoglycans, of which HS and hyaluronan constitute up to 90%,[7-9] are the main contributors to glycocalyx structure and function. This carbohydrate-rich layer with its associated proteins mediates many of the regulatory functions of the endothelium,[10] and the endothelial glycocalyx acts as the epicenter of the pathophysiology of various cardiovascular and renal diseases.[11] The IRI-induced shedding of the glycocalyx has been demonstrated in trauma patients[12] and dialysis patients,[13] rat cardiac IRI,[14] guinea pig cardiac IRI,[15] and vascular inflammation in rats.[16] Rehm et al[17] reported elevated plasma concentrations of syndecan-1 and HS as soluble markers of the damaged glycocalyx in patients after cardiac ischemia on cardiopulmonary bypass. This evidence shows that alteration of the glycocalyx is widely involved in endothelial damage caused by inflammation, and therapeutic strategies aiming at preserving its integrity may improve the outcome of IRI-related diseases.

Both anaphylatoxin (C3a, C5a)- and MAC-dependent mechanisms have been implicated in renal IRI.[18-21] De Vries et al[22,23] demonstrated both neutrophil-dependent and -independent effects of C5a in the pathogenesis of renal IRI. The MAC mediates neutrophil influx and inflammation, in addition to directly causing cellular damage and death.[19] Attempts to inhibit complement in IRI have targeted the classical and lectin pathways (C1 esterase inhibitor [C1-INH], C1s antagonist/antibodies), the lectin pathway alone (anti-MBL antibodies), or all 3 pathways (soluble complement receptor 1, anti-C5/C5a, C5a receptor antagonists).[2,24,25] C5 inhibition protects against IRI of the brain,[26] lung,[27] and myocardium[28] and renal[23] and allograft vasculopathy.[29] However, the effect of C5 blockade during renal IRI on shedding of the endothelial glycocalyx has not been investigated. In this study, we hypothesized that efficient inhibition of complement activation would attenuate renal IRI at least in part by preserving glycocalyx integrity. We tested this hypothesis in a mouse model of renal IRI by investigating whether (1) renal dysfunction is associated with complement activation and shedding of the endothelial glycocalyx, and (2) inhibition of complement using an antimouse C5 antibody would reduce glycocalyx damage and preserve renal function.

MATERIALS AND METHODS

Animals

Ten- to 12-week-old male C57BL/6 wild type mice were purchased from the Animal Resources Centre (Canning Vale, Western Australia). Mice were housed in an approved animal facility (Bioresources Centre, St. Vincent's Hospital Melbourne), and all experiments were approved by the Animal Ethics Committee of St. Vincent's Hospital Melbourne.

Warm Renal IRI

Mice were anesthetized by intraperitoneal administration of ketamine and xylazine and their core body temperature was maintained at 37°C during surgery by placing the mice on a heating pad. The kidneys were exposed by a midline abdominal incision and the renal pedicles were bluntly dissected. After right nephrectomy, ischemia was induced by occlusion of the left renal pedicle with a microvascular clamp (Roboz, Rockville, MD) for 22 minutes at 37°C in a temperature-controlled chamber. The clamp was removed after 22 minutes and the kidney was observed to confirm complete reperfusion. Sham-operated mice had right nephrectomy only without IR. All mice received 100 mL/kg of warm saline into the abdominal cavity during the procedure. The mice were recovered on a heat pad at 37°C. 24 hours after reperfusion, the mice were sacrificed by exsanguination, and blood and kidney samples were obtained.

Experimental groups (n = 7–8 per group) were as follows: (1) IRI mice treated with anti-C5 monoclonal antibody (mAb) BB5.1 (IRI/BB5.1), (2) IRI mice treated with isotype control antibody (IRI/isotype), and (3) sham control (Sham). 80 mg/kg (200–220 μL volume) of mouse antimurine C5 clone BB5.1 (a kind gift of Dr John Lambris, University of Pennsylvania, Philadelphia, PA) or isotype control mAb (BM4, mouse IgG1κ; CSL Ltd, Parkville, Australia) was administered intravenously just before ischemia. The sham mice did not receive any treatment.

Assessment of Renal Function

Renal function was assessed by measuring serum creatinine using a kinetic colorimetric assay based on the Jaffé method and analyzed on a COBAS Integra 400 Plus analyzer (Roche, Castle Hill, Australia) in accordance with the manufacturer's instructions. Serum urea was measured using Urea Assay Kit STA-382 (Cell Biolabs, San Diego, CA) as per the manufacturer's instructions.

Enzyme-linked Immunosorbent Assay for Complement C3b and C5a, Hyaluronan, Syndecan-1, and HMGB1

Plasma samples were analyzed using commercial ELISA kits for mouse C3b (HK216, Hycult Biotech, Uden, The Netherlands), C5a (DY2150; R&D Systems, Minneapolis, MN), hyaluronan (DY3614; R&D Systems), and syndecan-1 (75-138MS-S10; Alpco, Salem, NH), as per the manufacturer's instructions.

Plasma HMGB1 was measured using an in-house ELISA. In brief, a capture antibody, rabbit anti-HMGB1 (H9539; Sigma-Aldrich, Castle Hill, Australia) and detection antibody, HRP-conjugated rabbit anti-HMGB1 (ab128129; Abcam, Melbourne, Australia) were used, followed by SureBlue TMB Microwell Peroxidase Substrate (KPL 52-00-01, SeraCare, Milford, MA). Human HMGB1 full length protein, which shares 99% sequence homology with mouse HMGB1, was used as standard. Optical density was measured at 450/540 nm using a FLUOstar Omega microplate reader (BMG Labtech GmbH, Offenburg, Germany).

Immunofluorescence

Fresh-frozen tissue sections (5 μm) were fixed with acetone and incubated with rabbit anti-C3b/c FITC (Dako), rabbit anti-C9 Alexa 488 (Bioss Antibodies, Woburn, MA), mouse

anti-HS FITC (10E4 epitope, H1890; US Biologicals, Salem MA), rat antimouse VCAM-1 Alexa 488 (MCA2297; Bio-Rad, Raleigh, NC), rat antimouse Ly-6G FITC (Hycult Biotech), or rat antimouse F4/80 FITC (AbD Serotec). The slides were analyzed using a confocal microscope (Nikon A1R). Quantification of fluorescence intensity as raw integrated density ([RawIntDen], for C3b/c, C9, and HS with staining throughout the tissue) and mean gray values (for VCAM-1 with staining specifically in blood vessels) was performed using Image J software version 10.2 (National Institutes of Health).

Statistical Analysis

Results are presented as means ± standard error of the mean (SEM). Data plotting and statistical analysis were performed using Prism version 5.0 (GraphPad). Differences between the 2 groups were statistically evaluated using nonparametric t-test with Mann-Whitney *U* test (2-tailed) and a *P* value of <0.05 was considered to be statistically significant.

RESULTS

Treatment With Anti-C5 mAb BB5.1 Protects Against IR-induced Renal Injury

In mice subjected to right nephrectomy and 22 minutes left renal ischemia, severe loss of renal function was evident 24 hours after reperfusion. Serum creatinine (IRI/isotype group 190.0 ± 25.0 μmol/L vs sham 31.2 ± 1.7 μmol/L, $P = 0.002$) and urea (IRI/isotype 437.4 ± 12.2 mg/dL vs sham 73.8 ± 7.1 mg/dL, $P = 0.0006$) were both significantly elevated (Figure 1A and B). Treatment with the anti-C5 mAb BB5.1 just before ischemia (IRI/BB5.1 group) significantly reduced renal injury as seen by lower serum creatinine (121.0 ± 9.8 μmol/L, $P = 0.04$) and urea (316.3 ± 22.9 mg/dL, $P = 0.003$) compared with the IRI/isotype group, indicating that BB5.1 was protective against renal IRI in this model (Figure 1A and B).

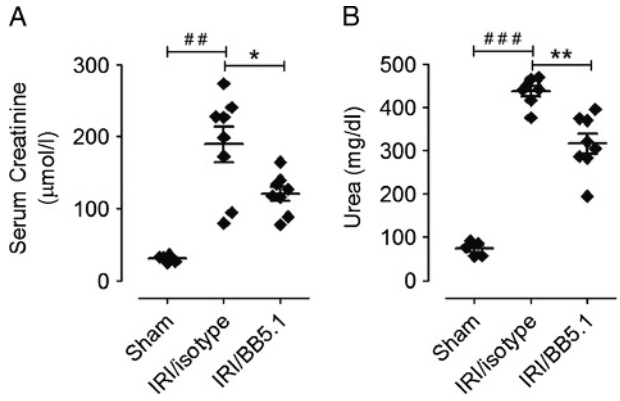

FIGURE 1. C5 blockade reduced renal dysfunction following IRI in mice. After right nephrectomy, the left kidney was subjected to 22 min ischemia. Twenty-four hours after reperfusion, renal function was assessed by measuring serum creatinine (A) and urea (B). Mice treated with 80 mg/kg BB5.1 (anti-C5) prior to ischemia showed significantly reduced serum creatinine and urea compared to isotype-treated mice. The data shown are mean ± SEM (n = 8). Significance was tested using the Mann-Whitney U test ($^{###}P < 0.001$ for sham vs isotype; $^{*}P < 0.05$, $^{**}P < 0.01$ for isotype vs BB5.1). IRI, ischemia-reperfusion injury; SEM, standard error of the mean.

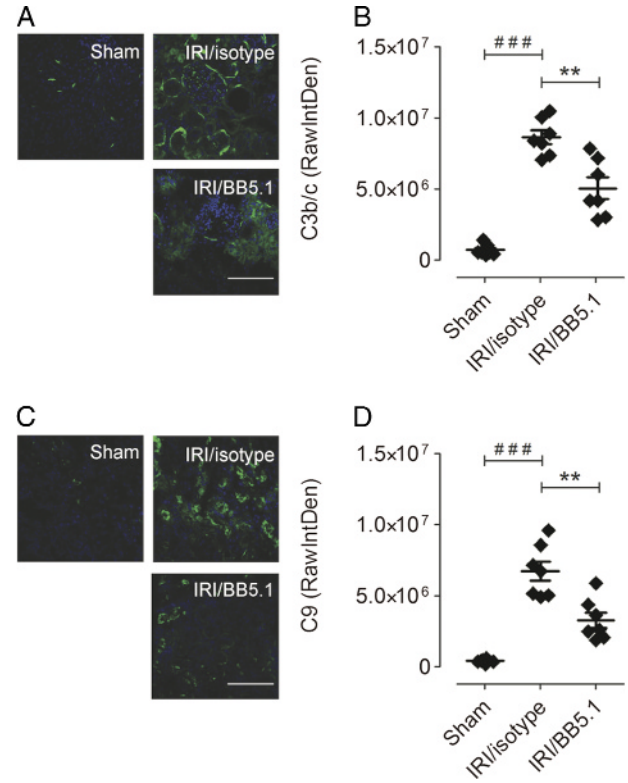

FIGURE 2. C5 blockade reduced renal IR-mediated complement activation and deposition. Twenty-four hours after reperfusion, kidney sections were analyzed for deposition of complement C3b/c (A) and C9 (C) using immunofluorescence staining/confocal microscopy, and fluorescence intensities were measured using Image J software (B and D). Nuclei were stained with DAPI. Tubular and glomerular epithelial and vascular endothelial C3b/c deposition (A) and tubular C9 deposition (C) were observed in isotype-treated mice. Treatment with BB5.1 reduced deposition of C3b/c (A and B) and C9 (C and D) compared with isotype control treatment. The data shown are mean ± SEM (n = 7–8). Scale bar, 50 μm. Statistical analysis was carried out using the Mann-Whitney U test ($^{###}P < 0.001$ for sham vs isotype; $^{**}P < 0.01$ for isotype vs BB5.1). IR, ischemia-reperfusion; IRI, IR injury; RawIntDen, raw integrated density; SEM, standard error of the mean.

Inhibition of C5 Activation by BB5.1 Treatment Reduces IR-induced Complement Activation and MAC Formation

Next, we investigated the effect of BB5.1 on complement activation during renal IRI. The degree of complement activation was measured using 2 methods: tissue deposition of C3b/c and C9 (as a measure of the MAC) and analysis of systemic C3b and C5a levels. Immunofluorescence/confocal analysis of kidney sections from sham-operated mice exhibited minimal C3b/c (Figure 2A) and C9 (Figure 2C). Sections from IRI/isotype mice showed significant deposition of C3b/c on the basement membrane of tubular and glomerular epithelium and vascular endothelium (Figure 2A) and of C9 on the basement membrane of tubular epithelium and vascular endothelium (Figure 2C). Deposition of C3b/c ($P = 0.004$) and C9 ($P = 0.004$) was significantly reduced in IRI/BB5.1 mice compared with IRI/isotype mice (Figure 2A–D). These results indicate the involvement of C3 and the terminal pathway of complement (C5b-9) in renal IRI.

We next determined systemic complement activation products by measuring plasma C3b and C5a. The C3b and C5a

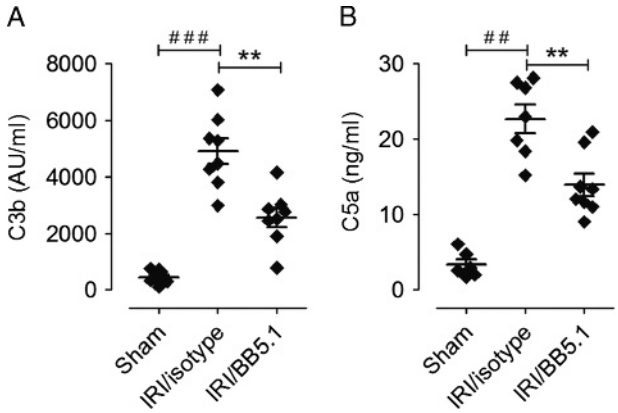

FIGURE 3. C5 blockade reduced the elevation of plasma C3b and C5a following renal IR. Twenty-four hours after reperfusion, plasma C3b (A) and C5a (B) were significantly higher in isotype-treated mice compared to sham-operated mice. Treatment with BB5.1 significantly attenuated this increase. The data shown are mean ± SEM (n = 7-8). Statistical analysis was carried out using the Mann-Whitney U test ($^{\#\#}P < 0.01$, $^{\#\#}P < 0.001$ for sham vs isotype; $^{**}P < 0.01$ for isotype vs BB5.1). IR, ischemia-reperfusion; IRI, IR injury; SEM, standard error of the mean.

levels in IRI/isotype mice were significantly increased 24 hours after reperfusion (C3b: 4913.0 ± 460.1 AU/mL vs sham 455.0 ± 81.5 AU/mL, $P = 0.0002$; C5a: 22.7 ± 2.0 ng/mL vs sham 3.4 ± 0.7 ng/mL, $P = 0.001$) (Figure 3A and B). Consistent with its effects on complement tissue deposition, BB5.1 treatment significantly decreased plasma C3b (2559.0 ± 340.1 AU/mL, $P = 0.001$) and C5a (14.0 ± 1.5 ng/mL, $P = 0.006$) levels compared with the IRI/isotype group (Figure 3A and B).

Blockade of MAC Assembly by BB5.1 Attenuates IR-induced Endothelial Activation and Inflammation

Alterations in renal endothelial function contribute to a reduction in renal blood flow and also influence vasodilation, coagulation, and inflammation following IR (reviewed in Basile and Yoder[30]). Therefore, we investigated the activation of the renal endothelium by assessing expression of the adhesion molecule VCAM-1 by immunofluorescence staining and confocal analysis. Expression of VCAM-1 was significantly upregulated ($P = 0.0006$) in the IRI/isotype group compared with sham-operated mice (Figure 4A and B) and correlated well with complement activation and deposition (with tissue C3b/c: $r^2 = 0.86$, $P = 0.003$; tissue C9: $r^2 = 0.9$, $P = 0.001$; data not shown). In contrast, expression of VCAM-1 was significantly reduced ($P = 0.003$) in samples from IRI/BB5.1 mice compared with IRI/isotype mice (Figure 4A and B) and correlated well with complement deposition after BB5.1 treatment (with tissue C3b/c: $r^2 = 0.77$, $P = 0.01$; tissue C9: $r^2 = 0.78$, $P = 0.009$; data not shown). Taken together, these data indicated that the inhibition of complement by BB5.1 reduced endothelial activation and preserved endothelial integrity.

Plasma HMGB1 levels, measured as important mediators of cellular activation and inflammation, were significantly increased after IR in the isotype-treated mice (31.3 ± 1.7 ng/mL vs sham 6.4 ± 0.9 ng/mL, $P = 0.0006$) (Figure 4C). BB5.1 treatment resulted in a significant reduction in plasma HMGB1 (14.8 ± 2.0 ng/mL, $P = 0.0003$ vs IRI/isotype) (Figure 4C).

Terminal Complement Inhibition by BB5.1 Prevents IR-induced Glycocalyx Damage

To evaluate the role of complement and renal IRI in the glycocalyx damage, expressions of vascular HS, syndecan-1

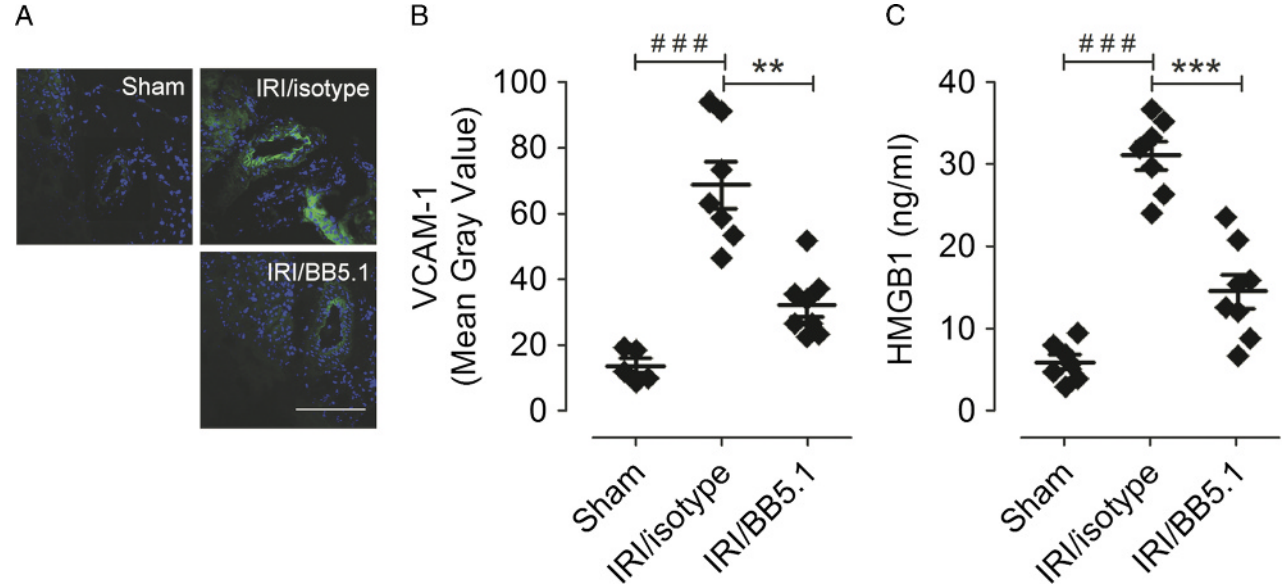

FIGURE 4. C5 blockade reduced renal IR-induced endothelial activation and inflammation. A, Representative images of VCAM-1 expression on kidney sections 24 h after reperfusion. Scale bar: 50 µm. B, Expression of VCAM-1 was significantly lower in BB5.1-treated mice compared to isotype-treated mice ($^{\#\#}P < 0.01$ for sham vs isotype; $^{*}P < 0.05$, $^{**}P < 0.01$ for isotype vs BB5.1). The data shown are mean ± SEM ($n = 7–8$). C, Plasma levels of HMGB1 measured by ELISA. Compared with sham, plasma HMGB1 levels were significantly higher 24 h after reperfusion in isotype-treated mice. Treatment with BB5.1 significantly reduced plasma HMGB1 levels. The data shown are mean ± SEM (n = 7–8). Statistical analysis was carried out using the Mann-Whitney U test ($^{\#\#\#}P < 0.001$ for sham vs isotype; $^{**}P < 0.01$, $^{***}P < 0.001$ for isotype vs BB5.1). IR, ischemia-reperfusion; IRI, IR injury; SEM, standard error of the mean.

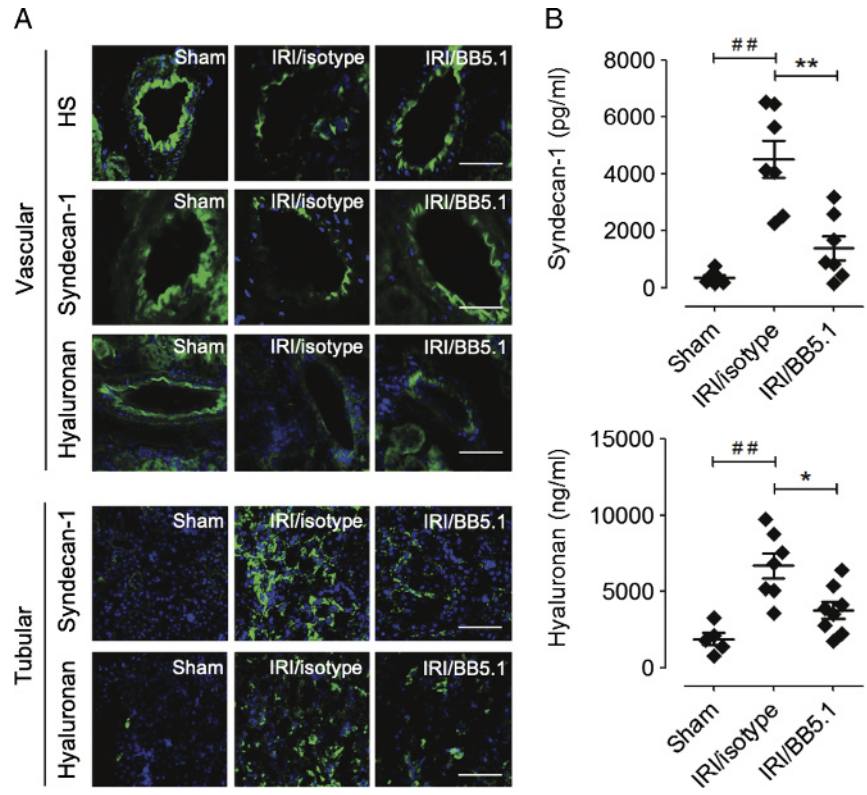

FIGURE 5. A, C5 blockade reduced renal IR-mediated endothelial glycocalyx shedding. Twenty-four hours after reperfusion, kidney sections were analyzed for expression of vascular HS, syndecan-1, and hyaluronan and accumulation of syndecan-1 and hyaluronan in the cortex and outer medullary regions of the kidney by immunofluorescence staining/confocal microscopy. Nuclei were stained with DAPI. Heparan sulfate expression was significantly reduced in the IRI/isotype group and preserved in the IRI/BB5.1 group. Scale bar: 50 μm. The data shown are mean ± SEM (n = 7–8). Statistical analysis was carried out using the Mann-Whitney U test ($^{\#\#\#}P < 0.001$ for sham vs isotype; $^{**}P < 0.01$ for isotype vs BB5.1). B, C5 blockade attenuated the increase in plasma syndecan-1 and hyaluronan following renal IR. Twenty-four hours after reperfusion, the plasma levels of syndecan-1 and hyaluronan were significantly higher in isotype-treated mice compared to sham control mice. Treatment with BB5.1 significantly attenuated this increase. The data shown are mean ± SEM (n = 7–8). Statistical analysis was carried out using the Mann-Whitney U test ($^{\#\#}P < 0.01$ for sham vs isotype; $^{*}P < 0.05$, $^{**}P < 0.01$ for isotype vs BB5.1). HS, heparan sulfate; IR, ischemia-reperfusion; IRI, IR injury; SEM, standard error of the mean.

and hyaluronan and tubular syndecan-1 and hyaluronan were measured by immunofluorescence and confocal staining. In the kidney of sham-operated mice, HS, syndecan-1, and hyaluronan were abundantly present on glomerular basement membrane and interstitium, tubular basement membrane, renal arterioles, the sub-intimal region, adventitia, and around smooth muscle cells of the media (Figure 5A). In contrast, vascular expression of HS, syndecan-1, and hyaluronan were greatly reduced, with a dramatic loss of staining intensity in kidneys from IRI/isotype mice (Figure 5A). Treatment with BB5.1 reduced this IR-induced loss of vascular HS, syndecan-1, and hyaluronan (Figure 5A). Interestingly, tubular accumulation of syndecan-1 and hylauronan was observed in the cortex and outer medulla in kidneys from IRI/isotype mice, possibly representing glycocalyx shed from the corticomedullary region of the kidney. This accumulation was reduced in BB5.1-treated mice (Figure 5A).

Since increased plasma syndecan-1 and hyaluronan levels are associated with glycocalyx breakdown, and they reflect different components of the glycocalyx (syndecan-1 as a core glycoprotein and hyaluronan as a loosely attached substance), we chose them as markers of the endothelial glycocalyx damage. Plasma levels of both markers were increased in IRI/isotype mice compared with sham-operated mice (syndecan-1 4510.0 ± 661.2 pg/mL vs sham: 349.0 ± 93.1 pg/mL, P = 0.001; hyaluronan 6658.0 ± 829.6 ng/mL vs sham: 1888.0 ± 414.5 ng/mL, P = 0.003) (Figure 5B). Treatment with BB5.1 significantly reduced circulating syndecan-1 (1393.0 ± 427.1 pg/mL, P = 0.007) and hyaluronan (3745.0 ± 553.2 pg/mL, P = 0.02) levels compared with isotype-treated mice (Figure 5B).

BB5.1 Treatment Reduces IR-induced Innate Cell Tissue Infiltration

To further characterize local renal inflammation upon IR, infiltrating Ly-6G-positive neutrophils and F4/80-positive macrophages were quantified by immunofluorescence and confocal staining. No or very few (1 or 2 cells per high-power field [HPF]) infiltrating neutrophils and macrophages were found in sham-operated kidneys (Figure 6A–D). Renal IRI induced significant recruitment of neutrophils (IRI/isotype 59.0 ± 3.0 cells/HPF vs sham, P = 0.0008) (Figure 6A and B) and macrophages (63.0 ± 5.0 cells/HPF vs sham, P = 0.0009) (Figure 6C and D) within the tubular interstitium at the corticomedullary junction corresponding to the area of severe injury and complement deposition. Kidneys from the IRI/BB5.1 group showed a significant reduction in infiltration of neutrophils (24.0 ± 4.0 cells/HPF, P = 0.0009) (Figure 6A and B) and macrophages (29.0 ± 7.0 cells/HPF,

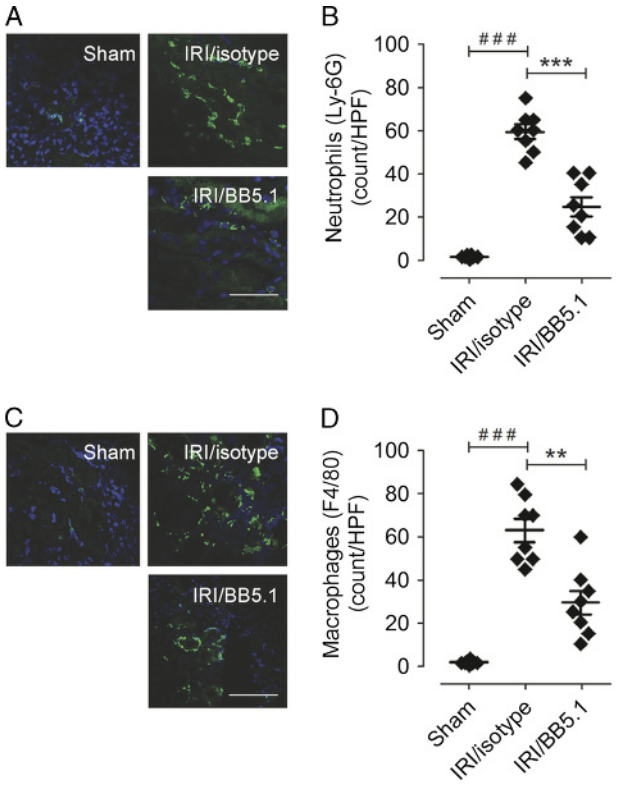

FIGURE 6. C5 blockade reduced recruitment of neutrophil and macrophage following renal IR. Twenty-four hours after reperfusion, kidney sections were analyzed for leukocyte infiltration by confocal staining, using Ly-6G/6C and F4/80 Abs for neutrophils (A, B) and macrophages (C, D), respectively. Nuclei were stained with DAPI. Scale bar: 25 μm (Ly-6G) and 50 μm (F4/80). The number of cells is expressed as count per HPF (B and D). Compared to sham, isotype-treated mice showed increased tubular interstitial infiltration of neutrophils (A) and macrophages (B). Treatment with BB5.1 significantly reduced infiltration of neutrophils and macrophages. The data shown are mean ± SEM (n = 8). Statistical analysis was carried out using the Mann-Whitney *U* test ($^{###}P < 0.001$ for sham vs isotype; $^{**}P < 0.01$, $^{***}P < 0.001$ for isotype vs BB5.1). HPF, high-power field; IR, ischemia-reperfusion; IRI, IR injury; SEM, standard error of the mean.

$P = 0.004$) (Figure 6C and D) compared with kidneys from the IRI/isotype group.

DISCUSSION

Acute ischemic injury of kidneys transplanted from donors after circulatory death is characterized by loss of integrity of the endothelial glycocalyx compared with kidneys from living donors.[31] The proposed mechanisms of glycocalyx shedding/degradation in the inflammatory transplant setting include endothelial cell activation and the action of reactive oxygen species and proteases released by activated neutrophils recruited to the graft.[32] Since complement activation contributes to these processes, we evaluated the effect of complement inhibition using an anti-C5 mAb on the loss of glycocalyx and impairment of renal function in a mouse model of renal IRI. C5 blockade prevented destruction of the glycocalyx and preserved kidney function, demonstrating a causal connection between terminal complement activation, glycocalyx damage, and IRI.

Complement activation is an early event in renal IRI and transplantation.[21,33] Although the relative involvement of the individual activation pathways remains a subject of debate, recent reports have indicated that local production of complement effector molecules[34-36] and the loss or abnormal expression of complement inhibitors[37] are possible contributors to complement activation following IRI. Complement activation products, such as C1q, C3a, C5a, and C5b-9, can directly activate and adversely affect endothelial function.[38] Upon reperfusion, an important and early reaction of the endothelium is the shedding of its native anticoagulant and anti-inflammatory surface layer, the glycocalyx. In addition, complement-mediated endothelial activation can potentially initiate and subsequently extend the initial tubular injury. This leads to the local release of damage-associated molecular patterns (eg, HMGB1) and other inflammatory mediators (eg, cytokines) and the expression of cell surface molecules to trigger activation of innate immune cells.[39,40] Therefore, effective inhibition of complement activation may offer tissue protection after reperfusion.

Reduced renal function following IRI in this study was associated with increased tubular complement C3b/c and C9 deposition and plasma C3b and C5a levels. In addition, we found significantly higher expression of VCAM-1, a marker of endothelial cell activation, and increased levels of circulating HMGB1. Extracellular HMGB1, secreted from necrotic or damaged cells or activated inflammatory cells, initiates potent innate immune responses in the pathogenesis of a range of inflammatory states, including IRI and transplant rejection.[41,42] Treatment with BB5.1 protected against IR-induced renal dysfunction, reduced complement activation and deposition, and attenuated endothelial activation and damage, reflected by reduced VCAM-1 expression and HMGB1 release. These results provide convincing evidence that complement activation is a critical effector mechanism that mediates postischemic renal inflammation and injury. Interestingly, blockade of C5 activation with BB5.1 also reduced the activation of C3, which is a preceding component in the complement cascade. We hypothesize that inhibition of the formation of C5a and C5b-9 reduced secondary cellular damage and inflammation including neutrophil recruitment, resulting in a reduction in ongoing C3 activation.

In normal conditions, glycocalyx components such as heparin/HS possess anticomplement properties through binding to various complement inhibitors.[43] Unregulated complement activation can induce shedding of this layer to create a proinflammatory and procoagulant endothelial surface, which is crucial for endothelial activation and dysfunction.[44,45] Furthermore, the anaphylatoxin C5a activates neutrophils to produce reactive oxygen and nitrogen species and release granular proteases, all of which can cause shedding of the glycocalyx.[46,47] Damage to the glycocalyx intensifies IRI in 2 distinct ways: loss of glycocalyx-related physiological functions from the cell surface, resulting in increased leukocyte- and platelet-endothelial interactions, inflammation, oxidative stress, and interstitial edema;[48] and release of shed glycocalyx fragments which can amplify the immune response by directly activating leukocytes and endothelial cells.[49] We therefore measured the tissue expression of HS, syndecan-1 and hyaluronan (presence of functional glycocalyx), and plasma levels of shed syndecan-1 and hyaluronan (degradation of glycocalyx). Indeed, we found a significant loss of the glycocalyx following IRI, indicated by reduced renal HS expression and elevated plasma syndecan-1 and hyaluronan

levels. Treatment with BB5.1 significantly reduced IR-induced tissue HS shedding and plasma syndecan-1 and hyaluronan, indicating preservation of the integrity of the glycocalyx. Together, these data support the notion that complement-mediated renal endothelial injury and dysfunction play a pivotal part in glycocalyx damage and renal dysfunction.

In line with previous reports, our study confirms that IRI can disrupt the integrity of the glycocalyx. Considerable evidence suggests that a variety of enzymes, increased oxidative stress, secondary inflammatory responses, and microvascular endothelial dysfunction contribute directly to the degradation of the glycocalyx (as reviewed in Lipowsky[47]). Activated inflammatory cells and resident macrophages produce oxygen/nitrogen species that can facilitate increased shedding of the glycocalyx via activation of sheddases and inhibition of endogenous protease inhibitors.[10,46,47,49,50] In addition, sustained endothelial activation induces increased release of enzymes such as heparanase and hyaluronidase that degrade the glycocalyx (as reviewed in Rabelink and de Zeeuw[51]). Matrix metalloproteinases, neutrophil elastase, thrombin, plasmin, tryptase, and cathepsin B, which are proteases released and activated under inflammatory conditions, cause shedding of the glycocalyx.[50] A limitation of the current study is that the precise mechanisms of glycocalyx shedding in renal IRI are not fully explored. Previous evidence suggests that the loss of glycocalyx function is reversible upon restoration of endothelial function.[52] Our results show that complement inhibition with anti-C5 attenuates endothelial activation and thereby limits glycocalyx destruction. Nevertheless, further experimental and clinical studies on glycocalyx shedding in renal IRI are warranted.

Complement-induced endothelial activation (evidenced by upregulated VCAM-1 expression), inflammation (HMGB1), and loss of glycocalyx might lead to extravasation of leukocytes, including neutrophils and macrophages, through the microvascular endothelium and subsequently further endothelial injury and dysfunction and tubular injury. This is particularly important in the medullary region as endothelial cells in this region express important surface markers for leukocyte activation.[53] Activated proximal tubular epithelial cells and leukocytes produce cytokines and chemokines that induce further cell infiltration and inflammation.[54] In this study, infiltration of neutrophils and macrophages has been documented in the corticomedullary junction of the kidney. These data provide evidence for the participation of innate cell infiltration and strongly implicate the involvement of C5a and C5b-9 in renal IRI. BB5.1 prevents generation of both C5a and C5b, and thereby C5b-9. Our data also show that inhibition of C5 using BB5.1 significantly reduced the influx of neutrophils as well as macrophages after IR. Together, these data emphasize that activation of C5 is central to the influx of neutrophils and macrophages and its inhibition with BB5.1 may also have contributed to the improved renal function by reducing cellular damage.

In summary, IRI-induced renal dysfunction was strongly associated with complement activation, complement-mediated endothelial glycocalyx damage, and innate immune cell infiltration. Inhibition of C5 significantly attenuated complement C3b/c and C9 deposition, endothelial activation, loss of the glycocalyx, and cellular infiltration. These data suggest an important role for the terminal pathway of complement in the injury process. Therefore, effective complement inhibition, by blocking the terminal pathway of complement, may prevent destruction of the glycocalyx and inflammation, thereby preserving kidney function following renal IRI.

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